

JC20 Rec'd PCT/PTO 12 MAR 2002

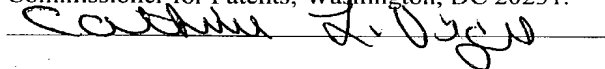
FORM PTO-1390 U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 10806-193
<b>TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371</b>		U.S. APPLICATION NO. (if known, sec. 37 CFR 1.5) <b>10/070787</b>
INTERNATIONAL APPLICATION NO. PCT/EP00/08984	INTERNATIONAL FILING DATE 13 September 2000	PRIORITY DATE CLAIMED 14 September 1999
TITLE OF INVENTION <b>Method for Increasing the Yield of Recombinant Proteins in Microbial Fermentation Processes</b>		
APPLICANT(S) FOR DO/EO/US NEUBAUER, Peter; LIN, Hong Ying		
<p>Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:</p> <ol style="list-style-type: none"> <li><input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371</li> <li><input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371</li> <li><input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</li> <li><input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date</li> <li><input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <ol style="list-style-type: none"> <li><input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</li> <li><input checked="" type="checkbox"/> has been transmitted by the International Bureau</li> <li><input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US)</li> </ol> </li> <li><input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</li> <li><input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) <ol style="list-style-type: none"> <li><input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</li> <li><input type="checkbox"/> have been transmitted by the International Bureau.</li> <li><input type="checkbox"/> have not been made; however, the time limit for making such amendment has NOT expired</li> <li><input checked="" type="checkbox"/> have not been made and will not be made</li> </ol> </li> <li><input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3))</li> <li><input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4))</li> <li><input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</li> </ol> <p>Items 11. to 16. below concern other document(s) or information included:</p> <ol style="list-style-type: none"> <li><input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98</li> <li><input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</li> <li><input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment</li> <li><input type="checkbox"/> A substitute specification</li> <li><input type="checkbox"/> A change of power of attorney and/or address letter</li> <li><input checked="" type="checkbox"/> Other items or information. Copy of published International Application No. WO 01/20016 A2 and A3, including International Search Report</li> </ol>		

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JC10 Rec'd PCT/PTO 11 MAR 2002

U.S. APPLIC. NO. (if known, see 37 CFR 1.50) <b>10/070787</b>		INTERNATIONAL APPLICATION NO. PCT/EP00/08984		ATTORNEY'S DOCKET NUMBER 10806-193	
				CALCULATIONS	PTO USE ONLY
17. The following fees are submitted.					
<b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b>					
<input checked="" type="checkbox"/>	Search Report has been prepared by the EPO or JPO		\$890 00		
<input type="checkbox"/>	International preliminary examination fee paid to USPTO (37 CFR 1.482)		\$710.00		
<input type="checkbox"/>	No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))		\$740 00		
<input type="checkbox"/>	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO		\$1040.00		
<input type="checkbox"/>	International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)		\$100 00		
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				\$ 890 00	
Surcharge of \$130 00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e))				\$	
Claims	Number Filed	Number Extra	Rate		
Total Claims	20 -20 =	0	x \$18 00	\$	
Independent Claims	1 -3 =	0	x \$84.00	\$	
Multiple dependent claim(s) (if applicable)			+ \$280.00	\$	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$ 890.00	
<input type="checkbox"/> Applicant(s) claim(s) small entity status, 37 C.F.R. 1.27. The fees indicated above are reduced by 1/2.				\$	
<b>SUBTOTAL =</b>				\$ 890 00	
Processing fee of \$130 00 for furnishing the English translation later than the <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				+	\$
<b>TOTAL NATIONAL FEE =</b>				\$ 890 00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)) The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) \$40 00 per property				+	\$
<b>TOTAL FEES ENCLOSED =</b>				\$ 890 00	
				Amount to be refunded	\$
				Amount to be charged	\$
a.	<input checked="" type="checkbox"/>	A check in the amount of \$890.00 to cover the above fees is enclosed			
b.	<input type="checkbox"/>	Please charge my Deposit Account No. in the amount of \$ to cover the above fees. A duplicate copy of this sheet is enclosed.			
c.	<input checked="" type="checkbox"/>	The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 04-1133.			
<p><b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b></p> <p><input checked="" type="checkbox"/> CUSTOMER NO. 24256 OR DINSMORE &amp; SHOHL 1900 Chemed Center 255 East Fifth Street Cincinnati, Ohio 45202 (513) 977-8200</p> <p><u>Holly D. Kozlowski</u> SIGNATURE</p> <p><u>30,468</u> REGISTRATION NUMBER</p> <p><u>Holly D. Kozlowski</u> TYPED OR PRINTED NAME</p> <p><u>12 March 2002</u> DATE</p>					

BASED ON FORM PTO-1390 (Rev. 10-2000)

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10806-193

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10/070787

Docket No. 10806-193

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*Catharine L. Vign*

JC10 Rec'd PCT/PTO 12 MAR 2002

**PATENT**

#3/a

**IN THE UNITED STATES PATENT & TRADEMARK OFFICE**

Applicant: Peter Neubauer et al : Paper No.:  
Serial No.: To be assigned : Group Art Unit:  
Filing Date: March 12, 2002 : Examiner:

For: **Method for Increasing the Yield of Recombinant Proteins in Microbial Fermentation Processes**

**PRELIMINARY AMENDMENT**

Box PCT  
Commissioner for Patents  
Washington, DC 20231

Dear Sir:

Prior to calculation of the filing fee and first action by the Examiner, please amend the present application as follows:

**In the Claims:**

Please amend claims 1-9 to read as follows:

1. (Amended) Method for increasing the yield of recombinant protein in a microbial fermentation process, wherein the concentration of a carbon / energy source in the culture is oscillatingly reduced or increased in short cycles.

2. (Amended) Method according to Claim 1, wherein the oscillations are generated by changing a dosage rate of a feed solution containing the carbon/energy source.

3. (Amended) Method according to claim 1, wherein the maximum duration of one cycle is 4 minutes, and the duration of a single phase of the cycle is a maximum of two minutes.
4. (Amended) Method according to claim 1, wherein the duration of one cycle is one minute, and the duration of a phase of the cycle is a maximum of 75% of the total cycle time.
5. (Amended) Method according to claim 1, wherein the carbon/energy source is added to the culture in such a manner as to cyclically vary the rate of addition of the substrate solution only during certain segments of the process.
6. (Amended) Method according to claim 1, wherein the dosage rate is controlled by cyclical activation and deactivation of the addition of the feed solution.
7. (Amended) Method according to claim 1, wherein glucose, glycerol, lactose, galactose, methanol, acetate, molasses, or starch is used as the carbon/energy substrate.
8. (Amended) Method according to claim 1, wherein, depending on the promoter used, IPTG, indolyl acrylic acid (IAA), lactose, arabinose, galactose, or methanol, if not already used as the energy source, is added to the culture to induce formation of the recombinant product.

9. (Amended) Method according to claim 1, wherein a temperature shift occurs at the time of the induction of the formation of the recombinant product.

Please add the following new claims 10-20:

--10. (NEW) Method according to claim 2, wherein the maximum duration of one cycle is 4 minutes, and the duration of a single phase of the cycle is a maximum of two minutes.--

--11. (NEW) Method according to claim 2, wherein the duration of one cycle is one minute, and the duration of a phase of the cycle is a maximum of 75% of the total cycle time.--

--12. (NEW) Method according to claim 2, wherein the carbon/energy source is added to the culture in such a manner as to cyclically vary the rate of addition of the substrate solution only during certain segments of the process.--

--13. (NEW) Method according to claim 3, wherein the carbon/energy source is added to the culture in such a manner as to cyclically vary the rate of addition of the substrate solution only during certain segments of the process.--

--14. (NEW) Method according to claim 2, wherein the dosage rate is controlled by cyclical activation and deactivation of the addition of the feed solution.--

--15. (NEW) Method according to claim 3, wherein the dosage rate is controlled by cyclical activation and deactivation of the addition of the feed solution.--

--16. (NEW) Method according to claim 2, wherein glucose, glycerol, lactose, galactose, methanol, acetate, molasses, or starch is used as the carbon/energy substrate.--

--17. (NEW) Method according to claim 3, wherein glucose, glycerol, lactose, galactose, methanol, acetate, molasses, or starch is used as the carbon/energy substrate.--

--18. (NEW) Method according to claim 2, wherein, depending on the promoter used, IPTG, indolyl acrylic acid (IAA), lactose, arabinose, galactose, or methanol, if not already used as the energy source, is added to the culture to induce formation of the recombinant product.--

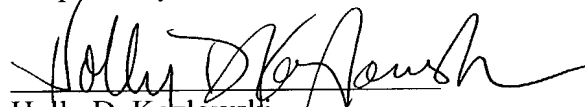
--19. (NEW) Method according to claim 3, wherein, depending on the promoter used, IPTG, indolyl acrylic acid (IAA), lactose, arabinose, galactose, or methanol, if not already used as the energy source, is added to the culture to induce formation of the recombinant product.--

--20. (NEW) Method according to claim 7, wherein, depending on the promoter used, IPTG, indolyl acrylic acid (IAA), lactose, arabinose, galactose, or methanol, if not already used as the energy source, is added to the culture to induce formation of the recombinant product.--

**REMARKS**

By the present Amendment, claims 1-9 are amended in accordance with customary U.S. patent practice and to omit the multiple dependency of the claims. A Version With Markings Showing Changes Made is attached. Claims 10-20 are added. Support for claims 10, 11, and 12 and 13 may be found in original claims 3, 4 and 5, respectively. Support for claims 14 and 15, 16 and 17, and 18-20 may be found in original claims 6-8, respectively. It is believed that these changes do not involve any introduction of new matter, whereby entry is believed to be in order and is respectfully requested.

Respectfully submitted,



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**VERSION WITH MARKINGS SHOWING CHANGES MADE****In the Claims:**

Claims 1-9 are amended as follows:

1. (Amended) Method for increasing the yield of recombinant [proteins] proteins in a microbial fermentation [processes, characterized by the fact that] process, wherein the concentration of [the] a carbon / energy source in the culture is oscillatingly reduced or increased in short cycles.
2. (Amended) Method according to Claim 1, [characterized by the fact that] wherein the oscillations are generated by changing [the] a dosage rate of [the] a feed solution containing the carbon/energy source.
3. (Amended) Method according to [Claims 1 and 2, characterized by the fact that] claim 1, wherein the maximum duration of one cycle is 4 minutes, and the duration of a single phase of the cycle is a maximum of two minutes.
4. (Amended) Method according to [any of Claims 1 through 3, characterized by the fact that] claim 1, wherein the duration of one cycle is one minute, and the duration of a phase of the cycle is a maximum of 75% of the total cycle time.
5. (Amended) Method according to [any of Claims 1 through 3, characterized by the fact that] claim 1, wherein the carbon/energy source is added to the culture in such a manner as to cyclically vary the rate of addition of the substrate solution only during certain segments of the process.



6. (Amended) Method according to [any of Claims 1 through 5, characterized by the fact that] claim 1, wherein the dosage rate is controlled by cyclical activation and deactivation of the addition of the feed solution.

7. (Amended) Method [pursuant to any of Claims 1 through 5, characterized by the fact that] according to claim 1, wherein glucose, glycerol, lactose, galactose, methanol, acetate, molasses, or starch is used as the carbon/energy substrate.

8. (Amended) Method [pursuant to any of Claims 1 through 7, characterized by the fact that,] according to claim 1, wherein, depending on the promoter used, IPTG, [or] indolyl acrylic acid (IAA), [or] lactose, arabinose, galactose, or methanol, [(if not already used as the energy source, is ) are] added to the culture to induce formation of the recombinant product.

9. (Amended) Method [pursuant to any of Claims 1 through 9, characterized by the fact that] according to claim 1, wherein a temperature shift occurs at the time of the induction of the formation of the recombinant product.

5/ppts

PATENT  
00362-PCT

**METHOD FOR INCREASING THE YIELD OF RECOMBINANT PROTEINS  
IN MICROBIAL FERMENTATION PROCESSES**

Industrial-scale production of recombinant proteins in bacteria takes place in fermenters. An increase in yield compared to laboratory-scale experiments done with flask shakers is achieved by increasing cell mass per volume. The batch-fed technique can achieve a high cell density. This is based on the growth-limiting addition of a nutrient source, whereby the carbon/energy source is generally limited (e.g. Riesenberg D. and Guthke, R., 1999, App. Microbiol. Biotechnol. 51, 422-430). For *E. coli* processes, this is normally glucose or glycerol. Alternatively, depending on the microorganism used and the process, however, other substrates are used, such as molasses, starch, peptone, lactose, methanol, and acetate. Highly concentrated feed solution can be added continuously, with the possibility to use various functions to define the addition of the substrate over a period of time, or linearly increasing and decreasing. Various functions are often combined within a single process. Alternatively, the nutritional solution can be added in pulses or at time intervals, with consumption of the nutrient or reduction of the amount of nutrient beneath a given concentration serving as the signal for the next pulse (e.g. Terasawa et al., 1990, EP 0 397 097 A1). The addition of the substrate solution can also be regulated using other parameters. Dissolved oxygen (DO-stat), pH (pH-stat), or the concentrations of carbon dioxide and oxygen in the exhaust determined on line (e.g. Kerns et al., Acta Biotechnol. 8, 285-289) can be used as control data, leading to cyclic dosing of the nutrient solution. In so doing, the concentration of the substrate is varied between a limiting and a nonlimiting concentration. Chen et al. (1997, Biotechnol. Bioeng. 56, 23-31) have measured increased plasmid stability when highly concentrated medium is periodically added to the batch-fed culture. In these procedures, one cycle can last several minutes or hours, which adversely effects product formation.

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Standard vectors for gene expression are the plasmids, which, in addition to the replication origin, normally contain the DNA sequence that encodes for the desired protein (product gene), as well as a selection marker that serves to guarantee the stable preservation of the plasmid during culture growth. The expression of the product gene is normally controlled via regulatory sequences, particularly regulable

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promoters. Expression of the product gene is activated, for example, by chemical inductors (substrates, substrate analogues), changes in cultivation temperature or other culturing conditions (pH value, salt concentration, degree of substrate concentration). In particular, induction can also occur by changing the limiting substrate, or by  
5 induction of the tac-promoter with lactose and switching from glucose feeding to lactose feeding (Neubauer et al., 1992, Appl. Microbiol. Biotechnol. 36, 739-744). Genes that provide the host cell with resistance to an antibiotic serve as selection markers for stable preservation of the plasmids in the host cells. Then, in the culture for the production of a recombinant protein, the corresponding antibiotic is normally  
10 added to kill off or inhibit the growth of plasmid-free cells that do not carry the resistance gene. Commonly used resistance gene/antibiotic pairs are  $\beta$ -lactamase/ampicillin, chloramphenicol-acetyltransferase / chloramphenicol, tetracycline resistance (tet)-operon/tetracycline, and kanamycin resistance gene /  
kanamycin.

15 Some of these resistance systems have the disadvantage that the antibiotic is deactivated by the resistance gene, as with ampicillin and chloramphenicol (e.g. Kemp G.W. and Britz M.L., 1987 Biotechnol. Techniques 1, 157-162). The consequence of this deactivation is that there is no obstacle to multiplication of  
20 plasmid-free cells in the culture. In addition, the proteins that mediate resistance can be released into the medium in the preparatory culture, accelerating the breakdown of the antibiotic. In these cases, the proportion of plasmid-free cells in the total culture can be increased. Moreover, no antibiotics are used in a large number of industrial processes for cost reasons or due to the additional expense incident to the subsequent  
25 cleaning, in which the remaining traces of the antibiotic or its deactivated form must be removed. A certain proportion of plasmid-free cells appears in such processes, as well.

While plasmid-free cells often only have a small growth advantage in the growth  
30 phase, in many cases, after product formation has begun, reduction in the growth rate of plasmid-containing, producing cells occurs, thus causing the culture to be overgrown by the plasmid-free cell population. The accumulation of plasmid-free cells has the disadvantage of reducing the relative proportion of the product in the

total cell mass, and, depending on the decomposition and cleaning methods chosen, making these postfermentation steps more difficult.

When constructing the vector, it is possible to limit these adverse effects, e.g. through  
5 selection of the resistance gene, the use of alternative, antibiotic-independent stabilization systems (Molin and Gerdes, WO84/01172), or by using modified antibiotics that break down more slowly; but the problematic resistances are still used. Moreover, none of the alternative systems is infinitely stable; stability can only be maintained for a certain time span.

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The invention stated in Patent Claim 1 is based on the problem of suppressing the overgrowth of plasmid-free cells after induction of recombinant product synthesis in batch-fed fermentations, particularly in industrial applications, without negative effects for product formation.

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The characteristics listed in Patent Claim 1 solve this problem by increasing / decreasing the concentration of the carbon/energy source in a cyclically oscillating pattern. This is achieved by changing the rate of addition of the feed solution that contains the carbon/energy source, e.g. by corresponding programming of the pump  
20 that doses the feed solution. This leads to sequential phases, in which the cells either have a limited amount of substrate available to them or none at all.

Contrary to the view held heretofore that oscillations adversely affect product formation in recombinant processes, targeted oscillations, with maximum cycle  
25 duration of four minutes, and individual phases of the cycle lasting a maximum of two minutes, surprisingly, have a positive influence on product yield. Cycle durations of approximately one minute (30 sec feeding, 30 sec pause) are particularly favorable.

The advantage of this method, which is principally applicable to all recombinant  
30 growth-limited processes in which the formation of the recombinant product is induced under carbon limitation, is that it is not necessary to add any further substances to the fermentation medium, that it is independent of the expression system used, and that it has no adverse effects on product formation. This procedure is particularly suited for batch-fed processes, in which a sugar, such as glucose, lactose,

arabinose, or galactose, or other organic carbon sources, such as methanol, glycerol, molasses, or starch as a limiting nutrient are added to the culture. The procedure is independent of the cultivation medium, and can be used for cultivation on mineral salt medium as well as complex media.

5

This method is not limited to *Escherichia coli* as host organism; rather, it can be used with all microorganisms, such as *Bacillus subtilis*, *Saccharomyces cerevisiae*, or *Pichia pastoris*, which are cultivated using carbon-limited batch feed. It is also independent of the induction system. However, it is particularly advantageous when

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using the tac-promoter.

The procedure is particularly advantageous when the expression of the gene product is strongly induced and growth of the producing cells is adversely influenced compared to a noninduced culture. In addition, this procedure is advantageous in processes in

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which the production phase is particularly long, e.g. in the periplasmatic expression of recombinant proteins or when the product formation phase is connected with a shift in temperature.

## Mode of Operation

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### *Strain and Plasmids*

*Escherichia coli* K-12 RB791 (F<sup>-</sup>, IN (rmD-rmE)1,λ<sup>i</sup>,lacI<sup>q</sup>L<sub>g</sub>; *E. coli* Stock Center, New Haven, USA) was used as the host. This strain was transformed with the plasmid

pKK177glucC (Kopetzki et al., 1989a), in which the α-glucosidase gene from

*Saccharomyces cerevisiae* is under the control of the tac-promoter. The plasmid

contains the β-lactamase gene as its selection marker. Additionally, a second system

was used, in which the plasmid pUBS520 (Brinkmann et al., 1989), which contains

the *dnaY* gene (Minor-tRNA *argU*, AGA/AGG), was transformed in addition to the

plasmid pKK177glucC.

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### *Cultivation Medium and Fermentation Conditions*

Glucose-ammonium-mineral salt medium (Teich et al., 1998, J. Biotechnol. 64, 197-210) was used for all cultivations. The initial concentration of glucose was 5 g l<sup>-1</sup>. The feed solution contained 200 g glucose kg<sup>-1</sup> and all components of the cultivation medium in the corresponding concentrations (Exception: (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 2.0 g l<sup>-1</sup>) and 10 ml l<sup>-1</sup> of the trace element solution (Holme et al., 1970), but no MgSO<sub>4</sub>. This was added during cultivation at 10ml of a 1 M MgSO<sub>4</sub> solution with OD<sub>500</sub>=9. Ampicillin (100 mg l<sup>-1</sup>) and kanamycin (10 mg l<sup>-1</sup>) were added both to the preparatory cultures and the fermentation medium. Polypropylene glycol 2000 (50 %) was used as an anti-foam agent.

Shake cultures on fermentation mineral salt medium, grown at 37°C, were used as the fermentation inoculum. All fermentations were carried out in 6 l Biostat ED Bioreactors with an initial volume of 4 L and a temperature of 35°C. The cultures were started as a batch culture. In this phase, the aeration rate and the stirring were regulated in a cascade mode in order to maintain a DOT of at least 20%. At the end of the batch phase, the DOT control was deactivated and the aeration rate and stirring speed were set at 2 vvm or 800 rpm. The pH value was regulated at 7.0 using a 25% ammonia solution. At the end of the batch phase, at a cell density of app. 2 g DCW l<sup>-1</sup> (OD<sub>500</sub>=9), the feeding pump was started at a constant rate of 53.2 g h<sup>-1</sup> (2.6 g glucose l<sup>-1</sup> h<sup>-1</sup>). The total amount of glucose added was the same in all cultivations, independent of the feed mode. Three different feeding strategies were tested: (A) continuous feeding (controlled cultivation), (B) intermittent feeding with a cycle of 1 minute (30 seconds on, 30 seconds off), (C) intermittent feeding with a cycle of 4 minutes (2 minutes on, 2 minutes off). The expression of the α-glucosidase gene was induced after adding 1 mM IPTG 3 h after feeding was started, and product formation was followed over a time span of app. 20 h after induction.

### *Analytical Methods*

Cell growth was followed by measuring the optical density at 500 nm (OD<sub>500</sub>). The microscopic cell count was further determined in a counting chamber (0.02 mm depth), and the dry cell weight (DCW) was determined (see Teich et al. 1998, J.

Biotechnol. 64, 197-210). The number of colony forming units (cfu) was determined by outcropping diluted samples on nutrient agar plates that were incubated for at least 3 days. Plasmid stability was then determined by overstepping these plates on selective agar with the replica plating technique. The relationship between DCW, OD<sub>500</sub>, and cell count was characterized as follows: 1g/l DCW corresponds to an OD<sub>500</sub> of 4.5±0.1 and a cell count of 1.8x10<sup>9</sup> ml<sup>-1</sup>. The glucose concentration was determined using a commercial enzyme kit.

The α-glucosidase concentration was determined after separating total cell samples in SDS gel (5% collection gel, 7% separation gel). Expression was carried out by scanning the product strip and quantification compared with a product standard placed in the gel in various concentrations.

### Results

*E. coli* RB791 pKK177glucC and *E. coli* RB791 pKK177glucC pUBS520 were cultivated in an agitation reactor using glucose-limited batch feed. After the first batch phase, constant feeding was started and, three hours after the start of feeding, the expression of the α-glucosidase gene was induced by adding 1 mM IPTG. After induction, there is an increase in the α-glucosidase concentration, whereby the specific concentration of the enzyme per cell reaches its maximum approximately 5 h after induction, and begins to reduce in longer cultivation (see Fig. 1c). The reduction of the specific concentration of α-glucosidase is due to the overgrowth of the culture with plasmid-free cells. These have an enormous growth advantage after induction, as the production of α-glucosidase adversely affects growth and also causes an inhibition of glucose uptake in the producing cells. This leads to accumulation of glucose in the culture medium. Cells present in the culture that do not contain the product gene are not influenced by the inductor IPTG, but rather continue to grow without limitation due to the high availability of glucose.

If the glucose solution is not added continuously, but rather in short-cycle pulses at intervals of about one minute (see Materials and Methods), the α-glucosidase will accumulate similarly to the constant feed after induction. However, overgrowth of the

culture by the plasmid-free cell population can be prevented depending on pulse duration (see Fig. 1d). This positive effect on the suppression of plasmid-free cells was not only obvious in the strongly expressing system shown in Fig. 1, but also in the weak expression of  $\alpha$ -glucosidase in the *E. coli* RB791 pKK177glucC system (Fig. 2, table 1). Moreover, pulse feeding had a slight positive influence on the synthesis rate in both cases after induction, and, in the first case, also on the stability of the product, more than 90% of which was present in the form of inclusion bodies. Definition of the cycle time is an important factor. In both examples shown, prolonging cycle time to 4 min causes a reduction in the amount of the product, and thus of the yield (see Fig. 1, 2 and Table 1). While the overgrowth of the culture by plasmid-free cells was reduced in this case as well, the longer cycle time leads to reduced product synthesis, or to increased breakdown.

Table 1: Productivity and Overgrowth by Plasmid-Free Cells in Glucose-Limited Batch-fed Cultures of *E. coli* RB791 pKK177glucC with and without PUBS520

Type of substrate added during batch-fed fermentation	$\alpha$ -glucosidase yield [mg/g biomass]		Plasmid-free cells [% of total population]	
	3 h post induction	20 h post induction	3 h post induction	20 h post induction
RB791 pKK177glucC pUBS520				
Constant feeding	37	30	2	72
Cycle 1 min	38	24	1	16
Cycle 4 min	37	6	2.5	60
RB791 pKK177glucC				
Cycle 1 min	10	9	10	10
Cycle 4min	6	4.6	15	6.7



**The figures show:**

**Fig. 1:** Batch-fed fermentations with *E. coli* RB791 pKK177glucC pUBS520 with induction by 1 mM IPTG. Comparison of continuous addition of glucose substrate solution (a-c; open symbols: without induction; filled symbols: with induction) with cyclic addition (d-f) of the same solution ( $\blacktriangle$ : cycle of 1 min;  $\nabla$  cycle of 4 min). (a,d) cell mass (DCW), (b,e) glucose concentration, (c,f) product formation (mg  $\alpha$ -glucosidase / g cell dry weight). The data shown represent a characteristic fermentation of 2 experiments performed for continuous addition and 1 experiment each for cyclic addition. Starting time for the addition of substrate solution (-----), induction with IPTG took place 3 h after feeding start (- - - - -).

**Fig. 2:** Batch-fed fermentations with *E. coli* RB791 pKK177glucC with induction by 1 mM IPTG. Comparison of continuous addition of the glucose substrate solution (a-c; open symbol: without induction; filled symbol: with induction) with cyclic addition (d-f) of the same solution ( $\blacktriangle$ : cycle of 1 min;  $\nabla$ : cycle of 4 min). (a,d) cell mass (DCW), (b,e) glucose concentration, (c,d) product formation (mg  $\alpha$ -glucosidase / g cell dry weight). For further explanations, see Fig. 1.

**Fig. 3:** shows the pump bowl scheme in a fermentation with a cycle of 1 min. A small section of the fermentation is shown, with the reaction of the dissolved oxygen (DOT, %, ----v----), as well as pump switching (0 = off, 1 = on, ---).

### Patent Claims

1. Method for increasing the yield of recombinant proteins in microbial fermentation processes, **characterized by** the fact that the concentration of the carbon / energy source in the culture is oscillatingly reduced or increased in short cycles.  
5
2. Method according to Claim 1, characterized by the fact that the oscillations are generated by changing the dosage rate of the feed solution containing the carbon/energy source.  
10
3. Method according to Claims 1 and 2, characterized by the fact that the maximum duration of one cycle is 4 minutes, and the duration of a single phase of the cycle is a maximum of two minutes.
- 15 4. Method according to any of Claims 1 through 3, characterized by the fact that the duration of one cycle is one minute, and the duration of a phase of the cycle is a maximum of 75% of the total cycle time.
- 20 5. Method according to any of Claims 1 through 3, characterized by the fact that the carbon/energy source is added to the culture in such a manner as to cyclically vary the rate of addition of the substrate solution only during certain segments of the process.
- 25 6. Method according to any of Claims 1 through 5, characterized by the fact that the dosage rate is controlled by cyclical activation and deactivation of the addition of the feed solution.
- 30 7. Method pursuant to any of Claims 1 through 5, characterized by the fact that glucose, glycerol, lactose, galactose, methanol, acetate, molasses, or starch is used as the carbon/energy substrate.
8. Method pursuant to any of Claims 1 through 7, characterized by the fact that, depending on the promoter used, IPTG or indolyl acrylic acid (IAA), or lactose, arabinose, galactose, or methanol (if not already used as the energy

source) are added to the culture to induce formation of the recombinant product.

- 5      9. Method pursuant to any of Claims 1 through 9, characterized by the fact that a temperature shift occurs at the time of the induction of the formation of the recombinant product.

ABSTRACT

The use of prior art methods for producing recombinant proteins in fed-batch fermentations often results, after the induction of the recombinant product synthesis, in an overgrowth of the culture due to plasmid-free cells and leads to a reduction of the specific product yield. The yield of recombinant proteins is thus increased by lowering or increasing, in a constantly brief manner, the concentration of the carbon/energy source in the culture. The oscillations are generated by altering the dosage rate of the feed solutions containing the carbon/energy source. This method is suited for all microorganisms which are cultivated using carbon-limited fed-batch.

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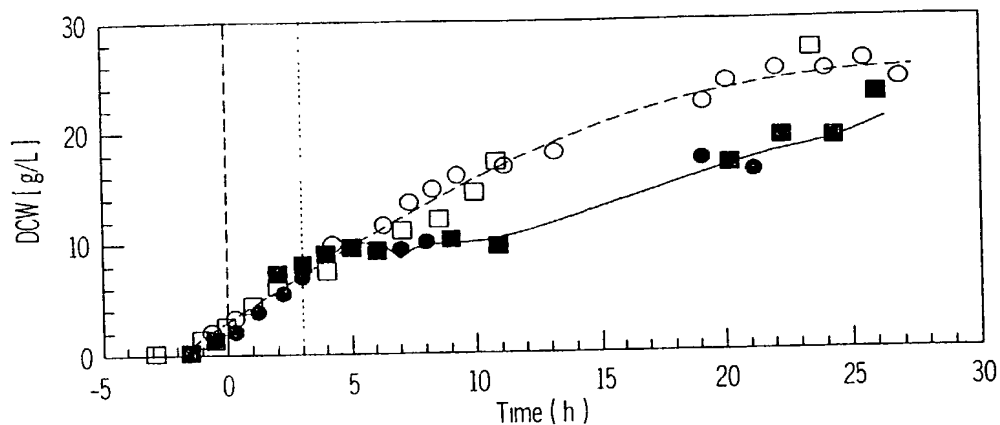


FIG. 1A

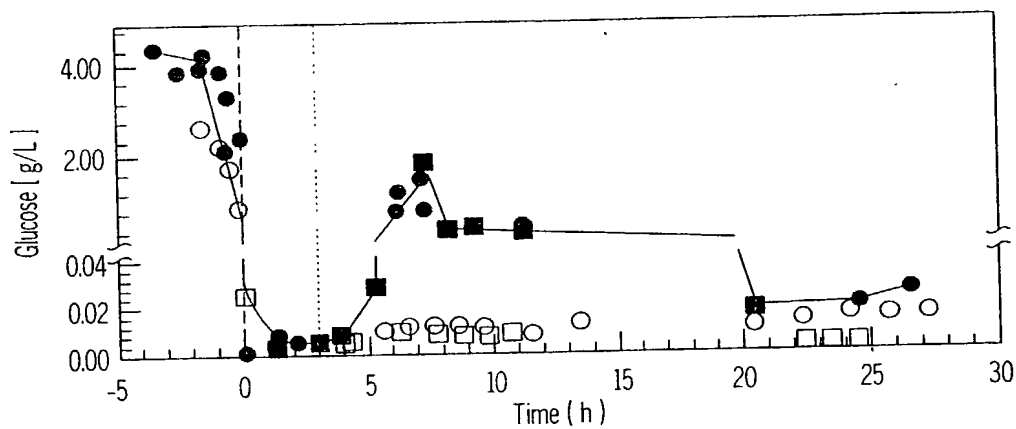


FIG. 1B

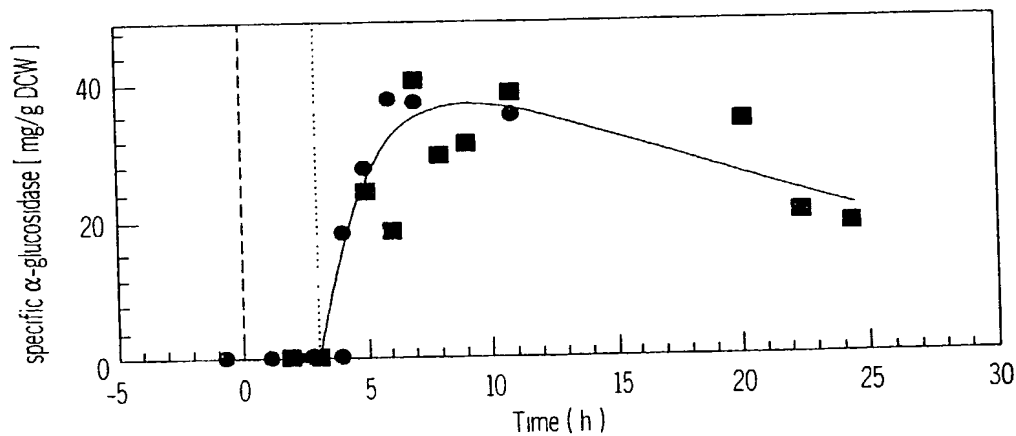


FIG. 1C

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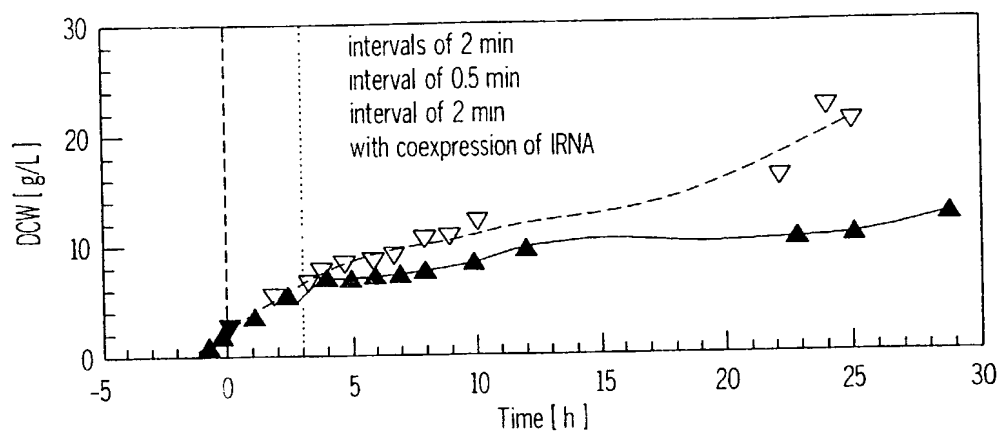


FIG. 1D

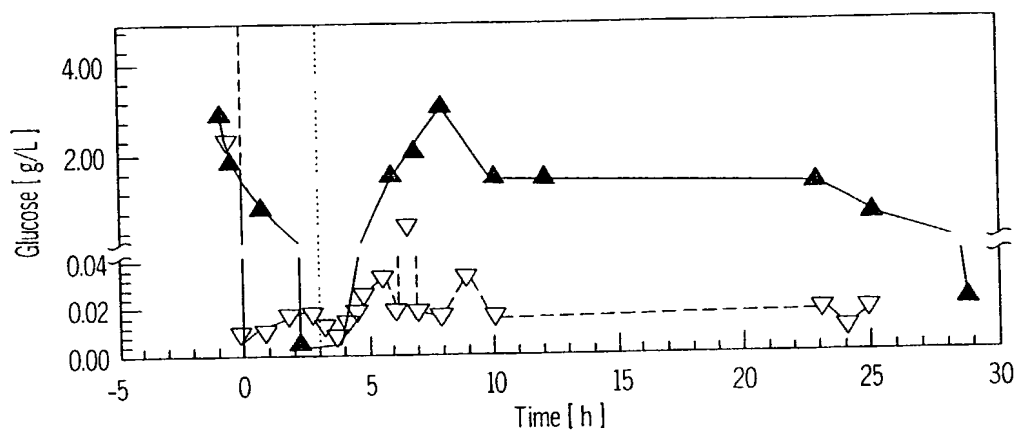


FIG. 1E

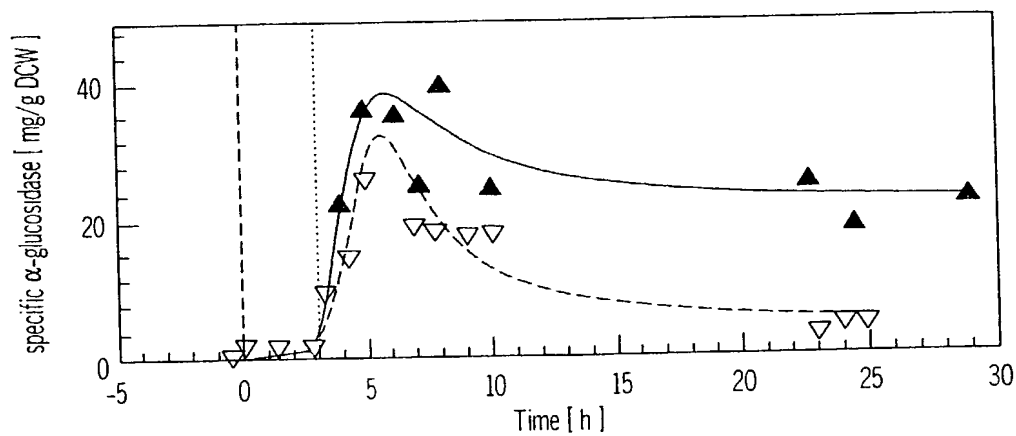


FIG. 1F

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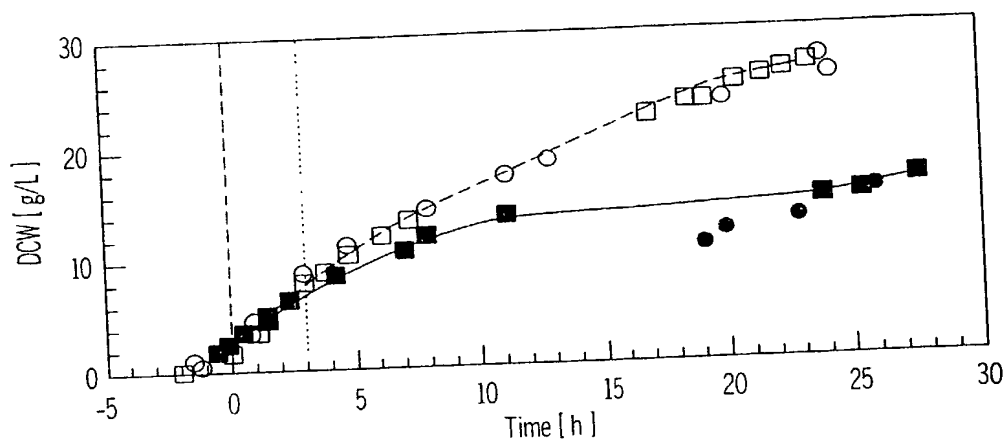


FIG. 2A

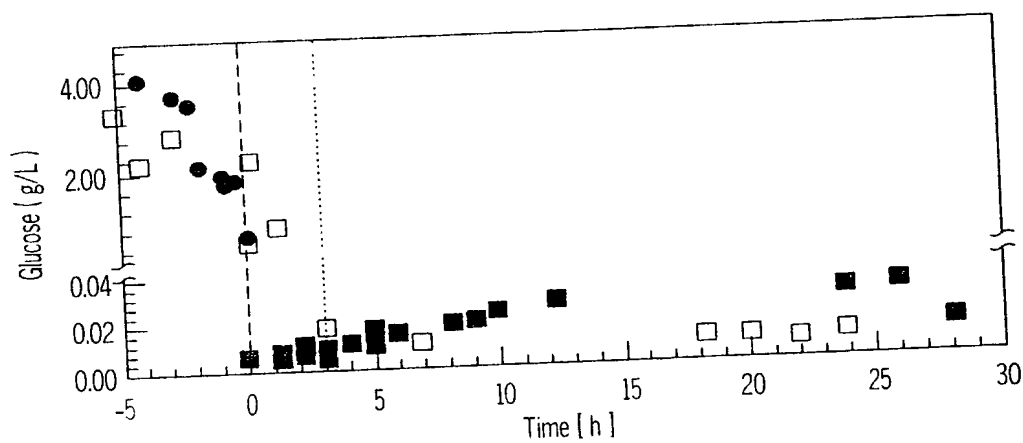


FIG. 2B

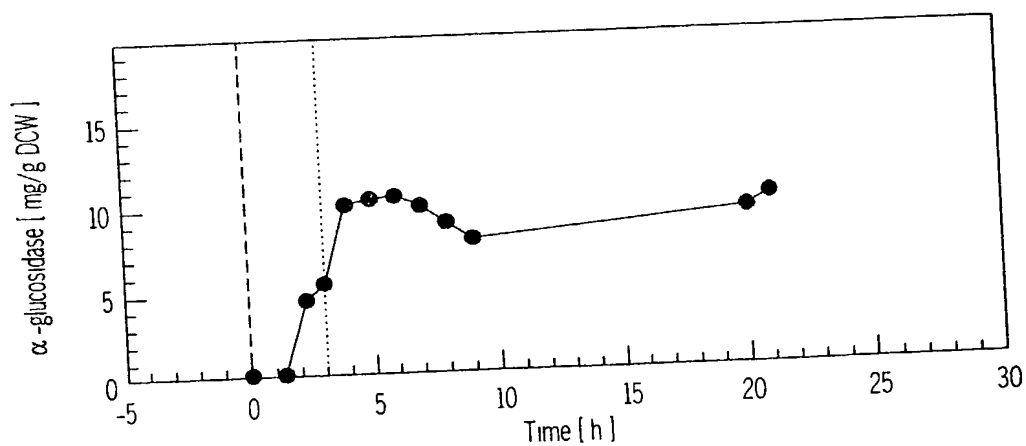


FIG. 2C

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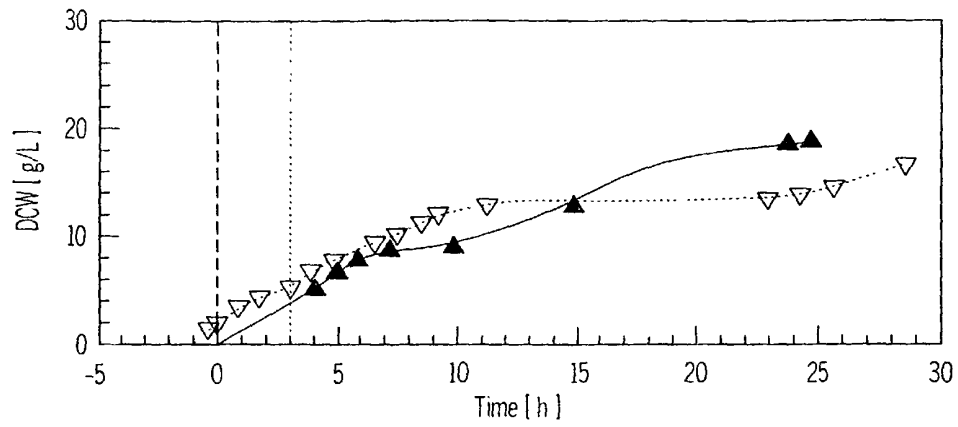


FIG. 2D

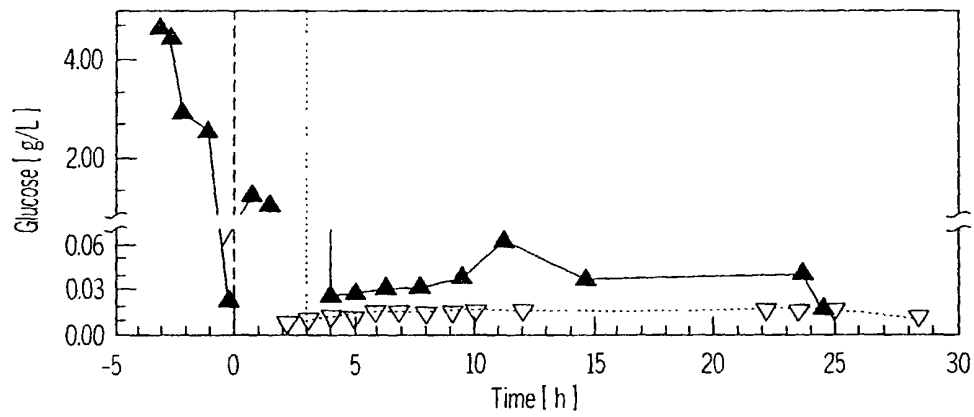


FIG. 2E

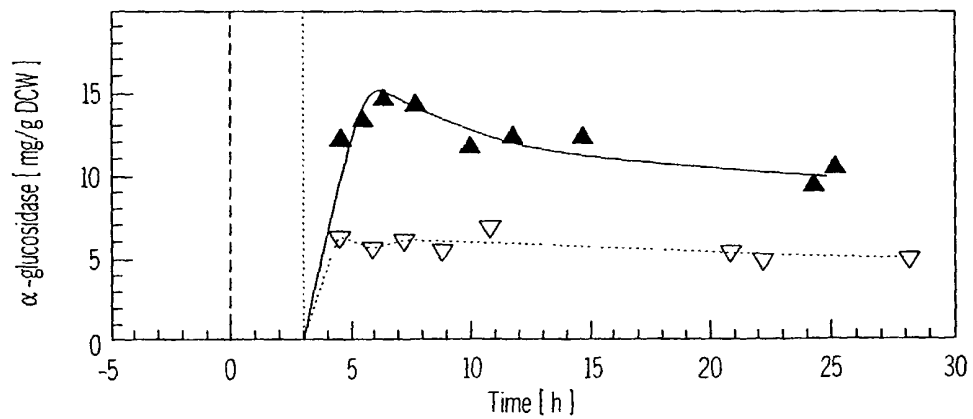


FIG. 2F



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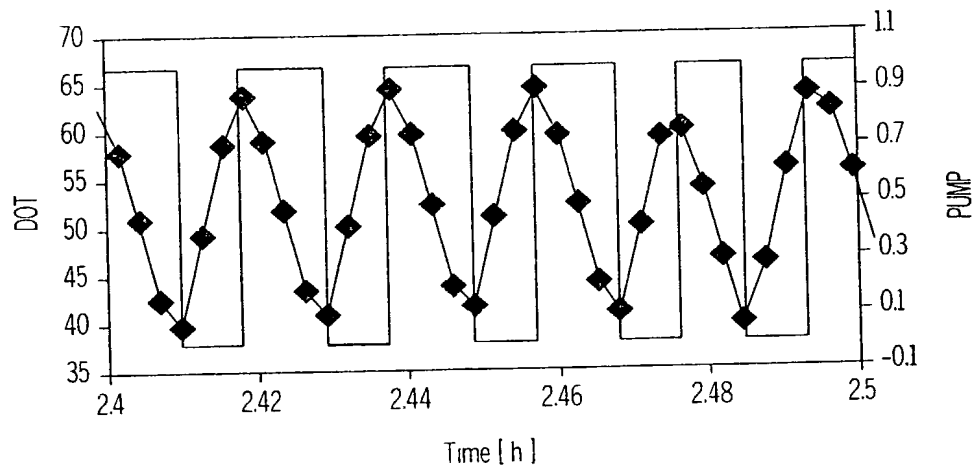


FIG. 3

**DECLARATION**  
**and**  
**POWER OF ATTORNEY**

**U.S. NATIONAL PHASE OF INTERNATIONAL APPLICATION**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **Method for Increasing the Yield of Recombinant Proteins in Microbial Fermentation Processes**, the specification of which was filed as International Application No. PCT/EP00/08984 on September 13, 2000,

- ☐ and was amended under Article 19 on \_\_\_\_\_  
(if applicable)
- ☐ and was amended under Article 34 on \_\_\_\_\_  
(if applicable)
- ☐ and was assigned U.S. Application Serial No. \_\_\_\_\_  
and was amended on \_\_\_\_\_  
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits and/or U.S. Provisional application priority benefits under Title 35, United States Code, §119 of any foreign application(s) or U.S. Provisional applications for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior U.S. Provisional or Foreign Application(s)			Priority Claimed	
Number	Country	Day/Month/Year Filed	Yes	No
199 43 919.2	Germany	14/09/99	X	

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulation, §1.56(a) which occurred between the filing date of the prior application and the PCT international filing date of this application:

(Application Serial No.)

(Filing Date)

(Status)

(patented, pending, abandoned)

⑩ I hereby appoint Holly D. Kozlowski, Registration No. 30,468; Ronald J. Snyder, Registration No. 31,062; James D. Liles, Registration No. 28,320; Lynda E. Roesch, Registration No. 29,696; Martin J. Miller, Registration No. 35,953; John V. Harmeyer, Registration No. 41,815; Geoffrey L. Oberhaus, Registration No. 42,955; John P. Colbert, Registration No. 45,765; Stephen S. Wentsler, Registration No. 46,403; and Ryan O. White, Registration No. 45,541, my attorneys, c/o Dinsmore & Shohl, 1900 Chemed Center, 255 East Fifth Street, Cincinnati, Ohio 45202 (513) 977-8200, with full power in each of them, of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

The undersigned hereby authorizes the above-named U.S. attorneys to accept and follow instructions from as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the undersigned and the aforementioned U.S. attorneys. In the event of a change in the firm or persons from whom instructions may be taken, the aforementioned U.S. attorneys will be so notified in writing by the undersigned.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

\*\*\*\*\*

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